MITOCHONDRIAL STRUCTURE IN PARAMECIUM AS REVEALED BY ELECTRON MICROSCOPY

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The structure of the mitochondria as revealed by electron microscopy has received considerable attention recently, and from these studies there have emerged several general descriptions of the ultrastructure of these cell particulates. Common to all the descriptions is the belief that mitochondria possess a membrane which is distinct from the internal matrix and which constitutes the external edge of the structure. The structures found in the interior of mitochondria have been variably interpreted as having ridges arising from the membrane projecting into a central matrix (Palade, 1953), parallel plates or membranes extending from side to side (Sjöstrand and Rhodin, 1953), concentrically arranged lamellae (Beams and Tahmisian, 1954), or parallel rods (Glimstedt and Lagerstedt, 1953). Our study of mitochondrial structure in *Paramecium,* based upon sections manipulated in a manner slightly different from those in most of the above studies, leads us to conclusions concerning structure which are different from any presented up to this time. A preliminary report of these has appeared (Powers, Ehret and Roth, 1954).

Materials and Methods

1. Organisms

Clonal cultures of two species of *Paramecium* were employed. The principal stocks examined were 51 VIII kk (Sonneborn) of *P. aurelia* and So 6 C (Chen) of *P. bursaria.* The organisms generally were used near the end of a growth cycle, very few food vacuoles being present in the cells at the time of fixation.

2. Fixation

The organisms, after having been concentrated by slow filtration through sintered glass, were fixed in 1% OsO_4, buffered at pH 7.4 with 0.045 *M* citrate-phosphate buffer (McIlvaine's), for 30 to 60 minutes at about 26° C. At the end of the fixation period the animals were carried through a series of fluids (without centrifugation) as follows: distilled water, 2 changes, 5 minutes each; 50% ethyl alcohol, 15 minutes; 75% ethyl alcohol, 30 minutes; one part absolute alcohol, one part methacrylate mixture, 30 minutes; methacrylate mixture, 3 changes, one hour each. The methacrylate mixture was 40% ethyl methacrylate and 60% n-butyl methacrylate with the hydroquinone inhibitor removed. After the third change, the plastic was irradiated with ultra-violet light for 12–16 hours at 40–45° C.; this treatment produced complete polymerization without the use of chemical catalysts.

3. Sectioning and mounting

Sections were cut with a glass knife in an International Minot thin-sectioning microtome equipped with a worm gear mechanism allowing a minimum thickness of 1/40 μ. Sections in the range 1/10 to 1/40 μ were used for observation. The solution used to float the sections was either 20% dioxane or 20% ethyl alochol. The sections were allowed to float on the solution for 10–20 minutes at 30–40° C. before being mounted on formvar-covered grids. Some of the sections were studied with no further preparations. Others were soaked briefly in toluene to remove the plastic. This treatment consisted of immersing the grid with the membrane-supported section for 20–30 minutes at room temperature.

4. Microscopy

An RCA type EMU-2A electron microscope was used. The microscope was equipped with the standard objective pole piece using a 60 μ objective aperture or with the wide-angle pole piece using rear focal plane objective aperture. The micrographs were taken at magnifications of 900–9200 diameters, further magnification being accomplished photographically.

Results

Identity of the particles

The particles studied here conform, in chemical characteristics, to descriptions of mitochondria given by other authors: they are osmiophilic and under certain physiological conditions they stain blue-green after Janus Green B, and pink after 2,3,5 triphenyl tetrazolium chloride. In addition they have been shown by Thomson (personal communication) to be associated with several oxidative enzymatic activities ascribed to mitochondria in other forms.

Size and shape

In crushed unfixed preparations under phase optics, the mitochondria appear as small spheroids about 0.7 μ in diameter. In 10% sucrose solution they also appear as spheroids. However, sections of mitochondria (Figs. 4–17) after osmic fixation appear to have been derived from cylinders with rounded ends, as well as from spheroids.

We believe that the spheroid bodies seen under phase optics in freshly crushed preparations may present the geometric shape of many of the particles in the living condition, because, as well as can be seen, the particles beneath the pellicle of the intact cell just before crushing appear spheroidal. If the spheroidal shape predominates in the living material, it is apparent that processing has caused a change in the shape of many of the particles from spheroids into cylinders and hyperboloids-of-one-sheet, with rounded ends, and with lengths about twice their widths. The volumes of the two structures are about the same (a cylinder 0.5 $\mu \times$ 1.0 μ, compared to a sphere 0.70–0.75 μ in diameter).

It is certain that fixation with OsO_4 results in some changes in size of organelles within these cells. The trichocysts, evident in Figures 2, 3, 4, and others, are larger than they are in the living cell. This fact is demonstrated by a comparison of Figure 1 with Figure 2. Figure 1 shows a group of trichocysts just

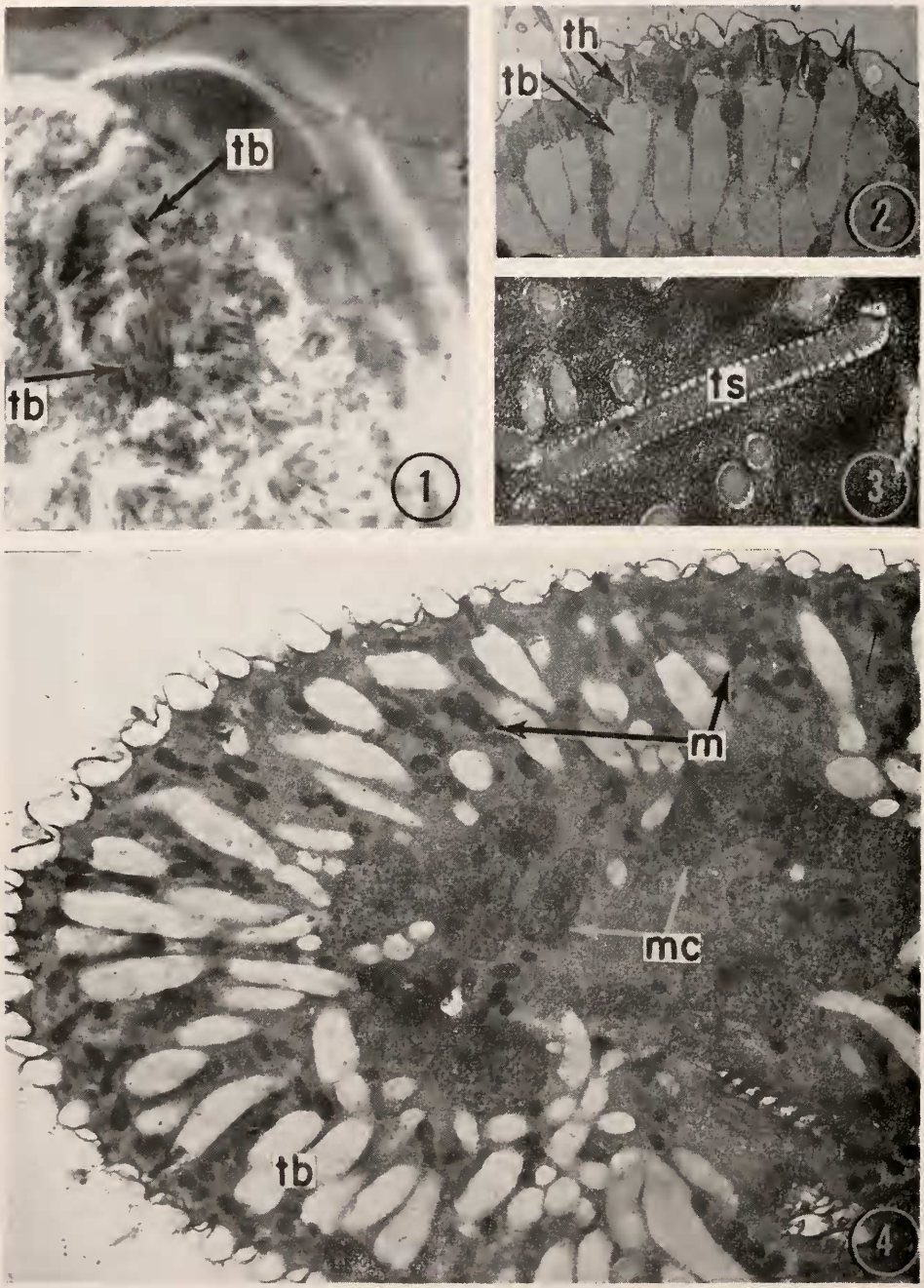


FIGURE 1. Phase contrast photograph of surface of compressed specimen of *P. aurelia* showing size of body of trichocyst relative to head in living condition. 1000 ×.

m, mitochondrion; mc, macronucleus; mcm, macronuclear membrane; mt, mitochondrial tubule; n, nucleolus; p, pore of mitochondrial tubule; pl, pellicle; pt, plastic; s, peritubular space; tb, trichocyst body; tc, trichocyst cap; th, trichocyst head; ts, trichocyst shaft ("exploded").

beneath the pellicle in a freshly crushed preparation. The tip length : body length ratio in this preparation, and in all others like it, is about 2 : 3. In the electron micrograph of sections of osmium-fixed material, the tip appears to retain its live dimensions, but the body, in all the preparations we have examined, is enlarged with a resulting ratio of 2 : 5 or more. In a few preparations complete shaft formation is seen even within the cell boundaries. So, as far as these structures are concerned, the fixation procedure is evidently not the best. In regard to others, however, the fixation procedure appears to be very good. The close adherence of the cytoplasm to the macronuclear membranes (Figs. 4, 5, 9) is in contrast to the situation observed after fixation of *Paramecium* by an agent such as warm Schaudinn's solution, which causes the appearance of an artificial vacuolar space separating the macronucleus from the cytoplasm. In regard to the mitochondria, it is probable that, in addition to the change in shape, some swelling of the structures occurred also, *i.e.*, in the undisturbed state the bodies may be slightly more compact.

Distribution

Figure 5 demonstrates that mitochondria in *P. aurelia* are, with certain exceptions, most abundant near the pellicle of the animal. The relationship between distribution of mitochondria and the reproductive stage of the cell will be discussed in a subsequent report.

Structure

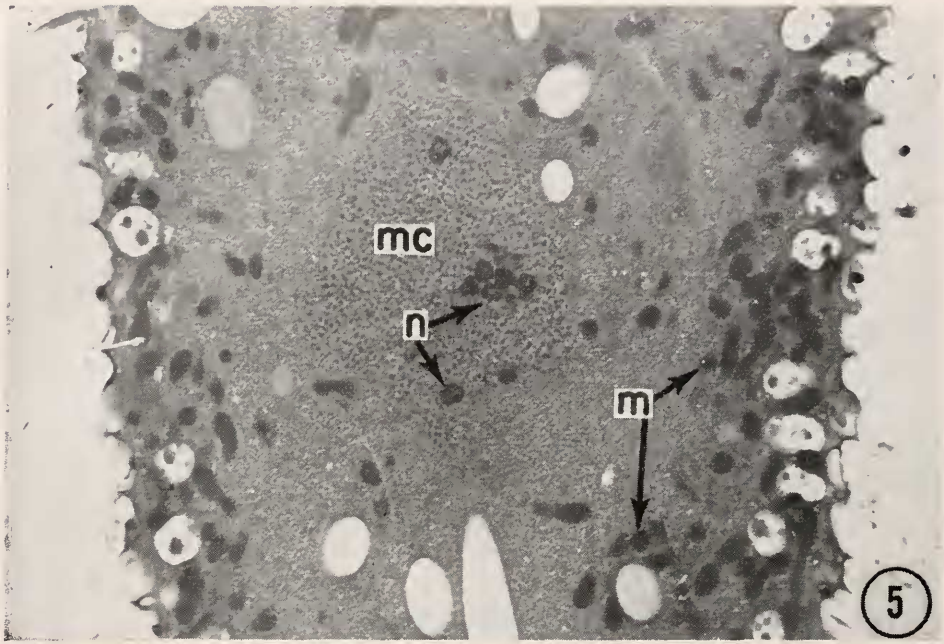
Figures 6–17 present the evidence for our idea of mitochondrial structure in *Paramecium*. Figure 6 is typical of the appearance of sections which are not soaked in toluene before examination. It may appear from this photograph that the mitochondrion is a sac bounded by a continuous membrane as postulated by others for metazoan and protozoan mitochondria. The central area (the "mitochondrial matrix" of other authors) appears clear and is approximately equivalent in density to the structureless ground substance representing the cytoplasm in these sections. Against this background, osmiophilic structures (the "microvilli" of Sedar and Porter, 1954) are found. Also in this section it is to be noted that the pellicle appears to be a double structure, and that the cytoplasm is homogeneous except for widely scattered dense granules.

Figure 7, from a section in which the plastic remains, demonstrates the same mitochondrial structure. It is presented to show in addition the appearance of the tip of the trichocyst and the surrounding cap when viewed in section with the plastic-in. This is to be compared with Figure 8, an adjacent section which was soaked in toluene for 30 minutes before examination. The tip of the trichocyst and the surrounding cap are sharply defined, in contrast to the fuzzy, ill-defined representation in Figure 7. Furthermore, no detail is visible in the space between the tip and the cap when the plastic is in, although threadlike structures extending between the tip and the cap are clearly visible after toluene treatment of the sec-

FIGURE 2. EMG (electron micrograph) of section of *P. aurelia*, demonstrating enlargement of trichocyst body relative to head after fixation with OsO_4. 5000 × Plastic removed.

FIGURE 3. EMG of fully extended trichocyst in cytoplasm. 10,000 ×. Plastic removed.

FIGURE 4. EMG of section of *P. aurelia*. Autogamous animal showing distribution of mitochondrial relative to the trichocysts and the macronuclear fragments. 4000 ×. Plastic removed.



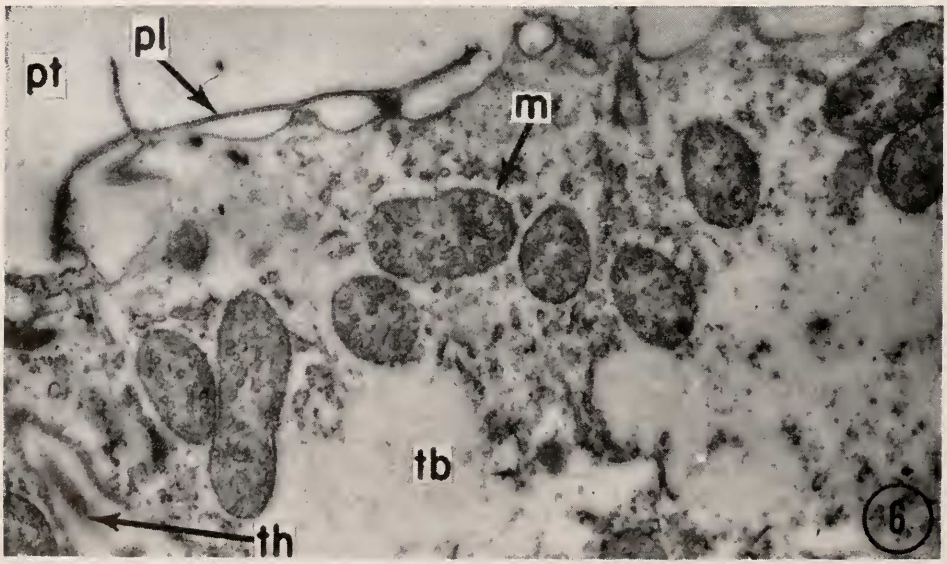


FIGURE 5. EMG of section of *P. aurelia* showing peripheral distribution of mitochondria in cytoplasm. Macronucleus containing nucleoli visible in center of section. 5000 ×. Plastic removed.

FIGURES 6 AND 7. EMGs of sections of *P. aurelia* with plastic remaining in. The cap, head, and body of the trichocyst are to be compared with corresponding structures in Figure 8. Note that the body of the trichocyst is hardly distinguishable from the plastic outside the pellicle. 20,000 × and 26,000 ×.

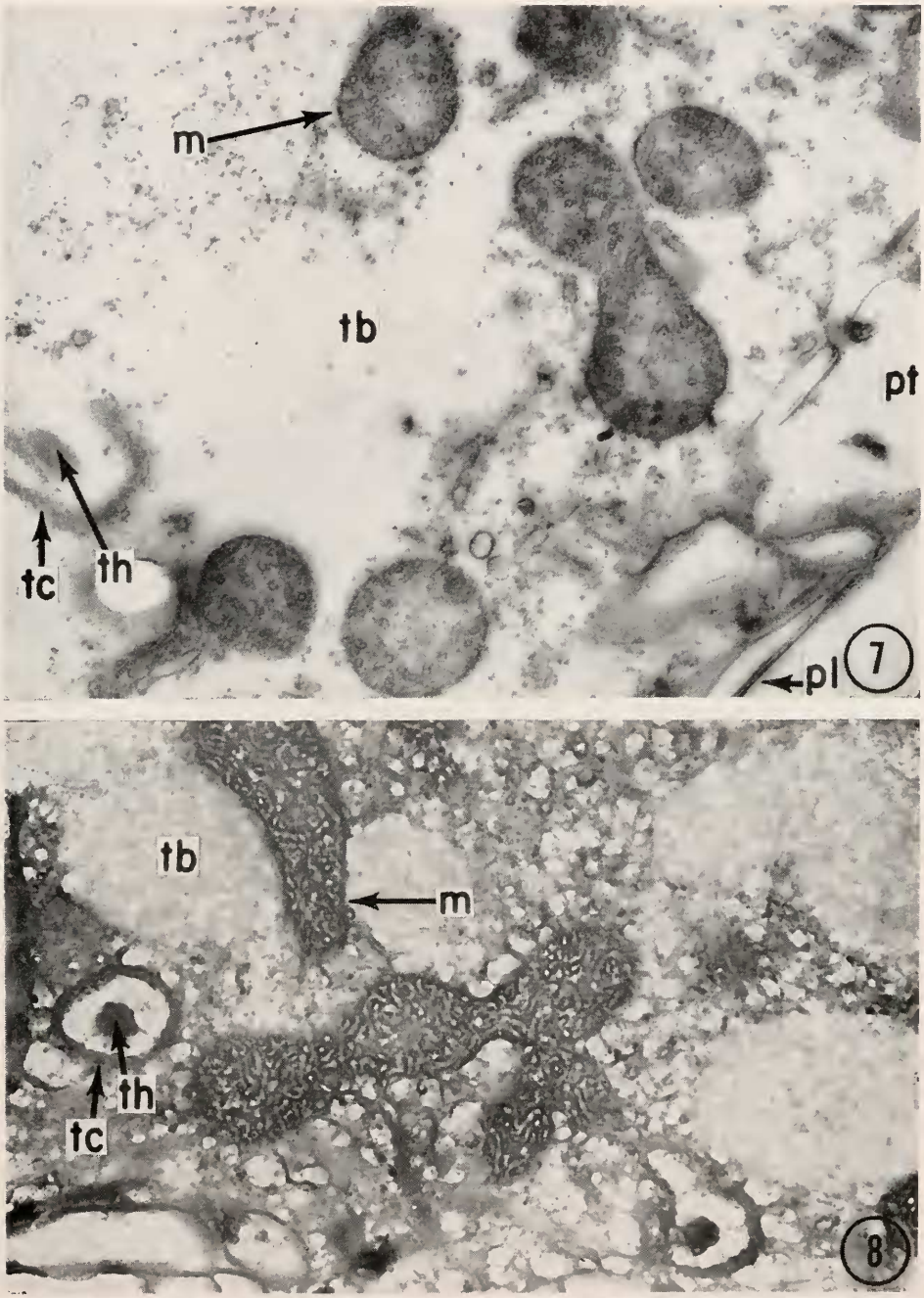


FIGURE 8. Section similar to those in Figures 6 and 7, except that toluene treatment preceded examination. This section came from the same block as those in the two previous figures. 26,000 ×. Plastic removed.

tion. The body of the trichocyst in Figure 7 is homogeneous and structureless; in Figure 8 the body is seen to have a grainy appearance. The ground substance of the cytoplasm in Figure 8, in contrast to Figure 7, is net-like, vacuolated, and contains particles of varying density (or osmium content). The frequency of occurrence of the densest particles indicates that they are identical with the cytoplasmic particles seen in Figure 7, but the net-like, less dense structures cannot be seen in Figure 7 because of masking by the plastic.

The mitochondria in Figure 8 are seen to consist of an osmiophilic material that is interrupted by numerous circular and elliptical interspaces. There is no external membrane distinct from this material. There is no clear central area but, rather, material that is less dense than some portions of the "outside edge" and material surrounding many of the interspaces. The more dense material about the spaces is continuous with the less dense material surrounding it.

Figures 9–17 are additional examples of sections through mitochondria demonstrating their ultrastructures. All the sections present essentially the same picture. There is an osmiophilic substance constituting the mass of the particle. This substance appears to be continuous throughout all sections except for very small interspaces. The interspaces appear as sharply defined circles, ellipses, or straight or contorted canals. Arrangements of the interspaces are not altogether random, for patterns are evident in the form of apparent concatenation of circles (Fig. 11), and whorled (Fig. 9) and anastomosing (Figs. 9, 10, 17) canals. All the sections show that the mitochondrion consists of this osmiophilic substance permeated by numerous small canals, irregularly but definitely arranged. The mitochondria of *P. bursaria* (Figs. 12, 13) appear more compact than those of *P. aurelia,* but otherwise show the same general structure.

The size of these interspaces was measured from greatly enlarged pictures. In a series of 30 circles, which were considered cross-sections of the interspaces, the mean diameter was 15 mμ with a standard deviation of 0.9 mμ. The range was 7.9 mμ to 25.2 mμ.

At first glance some of the sections (Figs. 16, 17) appear to show that the mitochondrion is surrounded by a membrane distinct from the interior, resembling the membrane of the macronucleus (Fig. 9). Closer examinations reveal, however, that the thick edge is clearly continuous with the material within the mitochondrion.

In many of the sections it can be seen that the interspaces are continuous with the cytoplasm, *i.e.*, an opening exists in the edge of the mitochondrion at that point. These holes are infrequent; they cannot be observed in most sections. However, numerous examples of them can be found and some are offered in Figures 14–17. Even when the plastic remains in the section, the structures visible in the matrix are occasionally seen to open to the outside (Fig. 7).

Discussion

The additional treatment of soaking the sections in toluene, which removes most of the plastic, reveals structure of the mitochondrion invisible in sections not so treated. On the basis of Roth's (1954) study of the effects of methacrylate on appearance of structure in sections, it is clear that the effect of the presence of the plastic is to mask certain detail in all parts of the cell, including the mito-

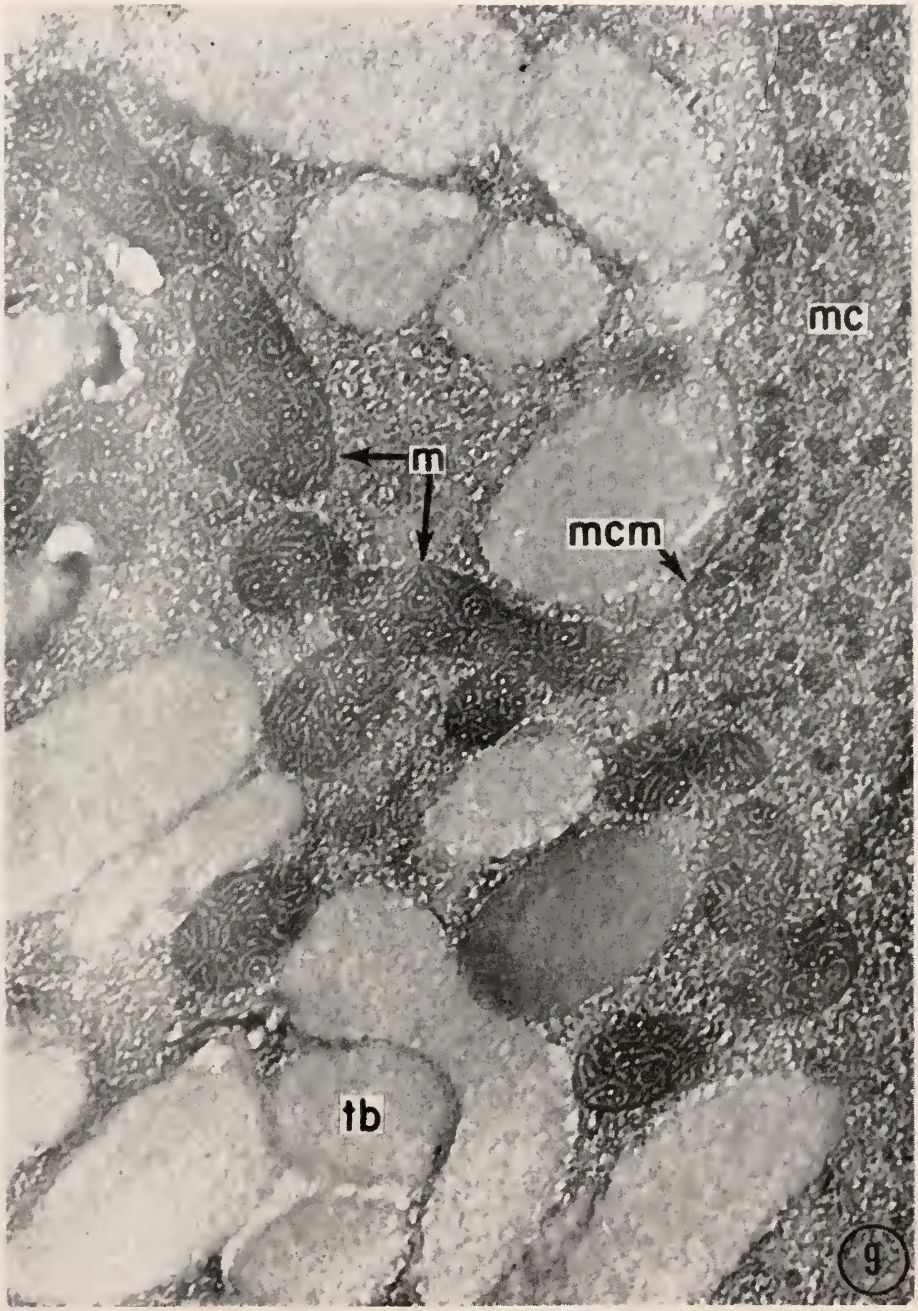


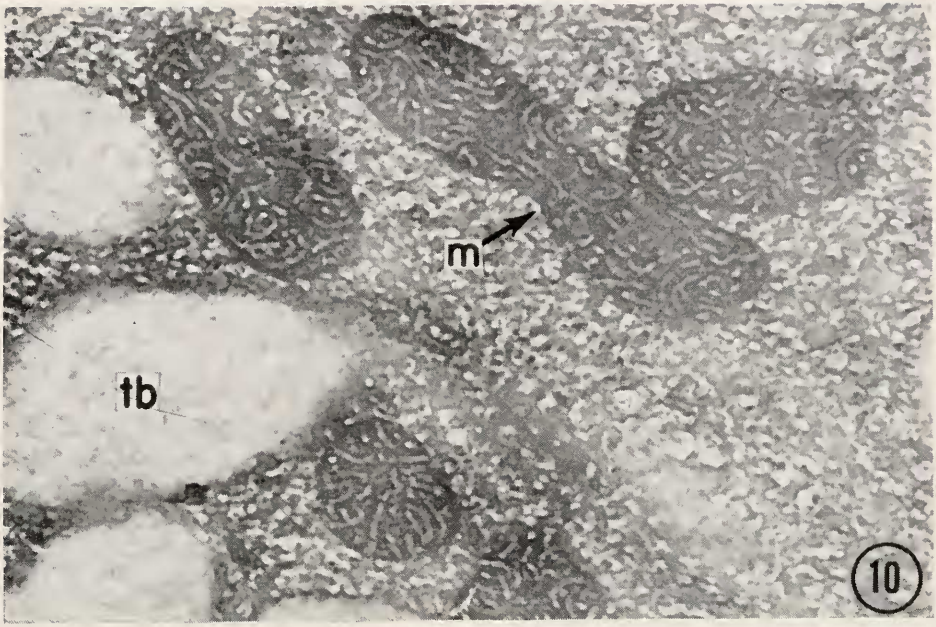
FIGURE 9. EMG of section of *P. aurelia*. On the right side is the macronucleus with its intact membrane. The details of mitochondrial structure are typical for *P. aurelia*. 42,000 ×. Plastic removed.

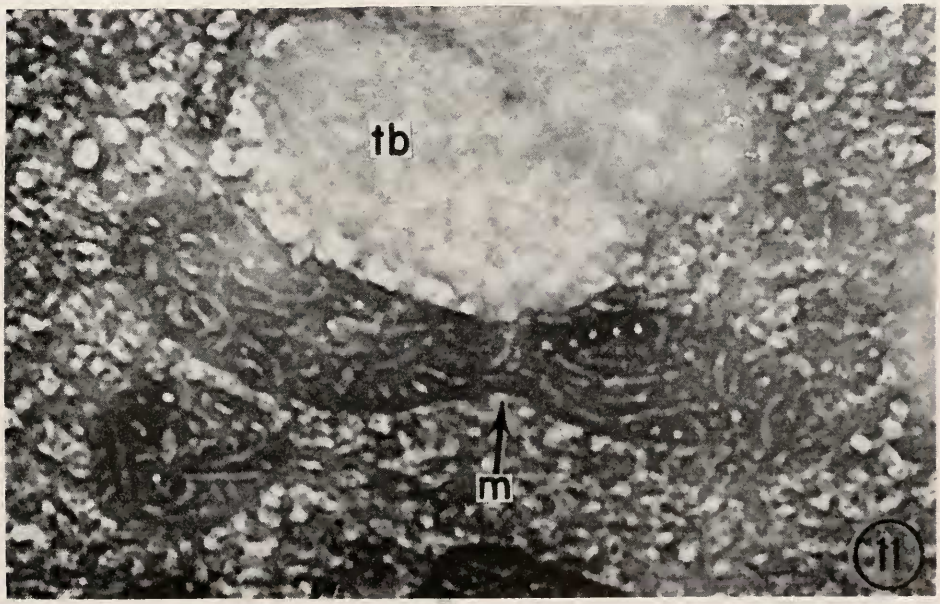
chondrion. All material of low electron scattering power ("density") is obscured by the plastic because it does not allow distinction between this low density material and those areas which appear as spaces in the sections prepared in the other manner. At the same time, the difference in effective density between the high density material and the low density material is greater, so that these areas are in sufficient contrast against the "structureless" background to be visible in the photograph (*e.g.,* some of the material immediately bordering the canals and the very dense granules of the cytoplasm). The result is that we see thin-walled "tubules" in the mitochondria and discrete, well-separated granules in the cytoplasm. The knowledge, then, that areas of low density are not distinguishable from those of no density and that areas of great density are the only ones seen when the plastic remains in the sections reconciles differences in appearance by the two techniques.

While removal of plastic may cause some displacement of ultrastructure (Hillier and Gettner, 1950), we propose that the structure of the mitochondrion is revealed only under these conditions. Removal of plastic from these sections results in cross-sections of cilia which seem collapsed to a slight extent because the double structure of the individual fibers reported by others (Fawcett and Porter, 1954) is recognized only with difficulty. But it should be noted that the change brought about by the removal of the plastic is a slight movement of structure, and this kind of change hardly accounts for the architecture observed in the sections of mitochondria after plastic removal. It is difficult to conceive that structure of such high degree of organization (comparable in all the details visible in sections before plastic removal) is the result of changes brought about by solvent action.

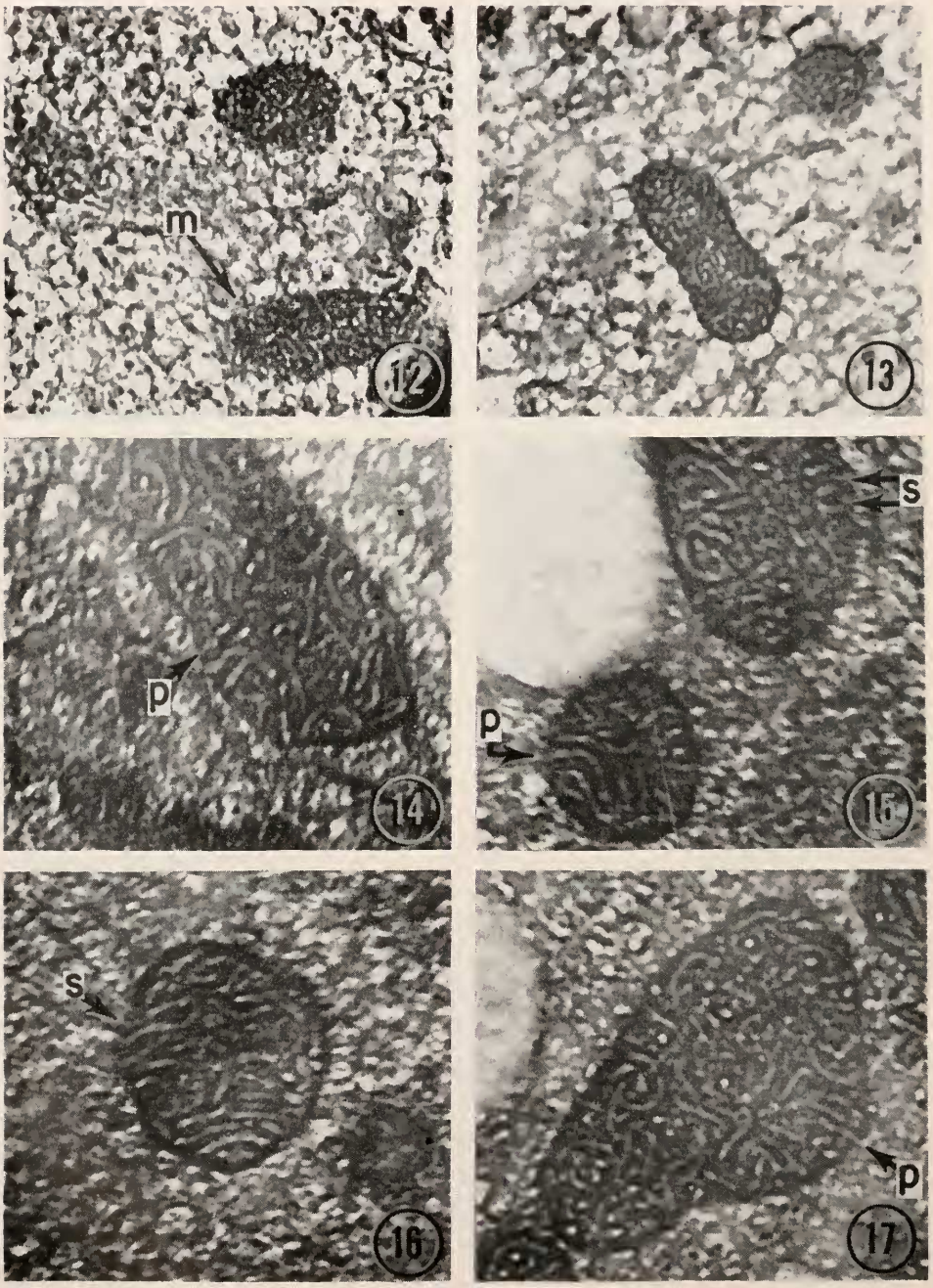
There are two other reports of structure of mitochondria of protozoa as revealed by electron microscopy, one in *Euglena* (Wolken and Palade, 1953) and the other in *Paramecium multimicronucleatum* (Sedar and Porter, 1954). These authors, using the generally accepted method of leaving the plastic in during examination, conclude that the mitochondria possess external membranes. In *Euglena* numerous ridges (called *cristae*) project from the "membranes" into an internal structureless matrix. The authors point out that this mitochondrion has a differentiated membrane similar to that observed in mitochondria of other animal cells. In *P. multimicronucleatum,* it is said, there are villi projecting from the external "membrane" into the internal matrix. The latter report is reconcilable with our observations in all respects, for, prior to being soaked in toluene, the sections show the same structure reported by these authors. In regard to the observation in *Euglena* (Wolken and Palade, 1953), we suspect that removal of the plastic might reveal that the matrix has real structure, and that this structure may have the same relationship to the "membrane" as that observed in our studies.

In other studies (Palade, 1953; Sjöstrand and Rhodin, 1953) mitochondrial membranes are described from sections of mammalian tissues in which the plastic remains during examination. Very clearly defined dense lines are visible about the particles, but close examination of many of the photographs shows that the membranes are frequently discontinuous and vaguely defined, and sometimes bear unexpected (for membranes) relationships to the structures found internally. In view of this and in view of our demonstration that plastic removal reveals





FIGURES 10 AND 11. EMGs of mitochondria of *P. aurelia* at high magnification showing typical structure. 69,000 × and 84,000 ×. Plastic removed.



FIGURES 12 AND 13. EMGs of sections of *P. bursaria* showing the same general mitochondrial structure as observed in *P. aurelia*. 26,000 ×. Plastic removed.

FIGURES 14–17. EMGs of sections of *P. aurelia*. Pores of mitochondrial tubules and peritubular space are shown. Figure 17 is an enlarged mitochondrion from Figure 9. 67,000 ×, 67,000 ×, 74,000 ×, and 67,000 ×. Plastic removed.

many details of structural interrelations not visible in other sections, the generalization that mitochondria have differentiated membranes is open to question.

Two general kinds of interpretation of the structure of the mitochondrion can be made on the basis of the observations reported here. The particle can be considered a continuum which is interrupted by numerous canals, the canals bearing the same relation to the substance of the particle as does the space made by a pin in a lump of clay to the clay itself. Or, it can be considered to be made up of a tightly packed collection of tubules (like hollow macaroni), the walls of which in some places fuse to form a continuum of osmiophilic substance. In other areas the tubular characteristic is retained.

Examination of the figures presented, especially in the regions of looser structure, leads us to favor the tubular interpretation. In this interpretation, the basic structure is a tubule with a lumen approximately 15 mμ in diameter. The innermost portion of the tubule wall appears very dense after osmium fixation; the outermost portion of the wall, except at the edge of the particle, is frequently less dense. These portions often are continuous with corresponding parts of adjacent tubules setting up a continuum not separable into component tubules. In places the tubules are evidently not continuous, forming what might be called the peritubular (or intertubular) space. At the edge of the particle this continuum is often dense. Infrequently, the tubules are seen to open through the dense edge of the particle, the lumen being continuous with the surrounding cytoplasm. The lumen itself is represented by a space in the photographs. Of course, this does not mean that this lumen is empty in the living condition; it does mean that any substance occurring there must be different from that of the wall of the tubule which reduces OsO_4 and becomes relatively dense.

This interpretation accounts for all details visible in both types of sections. With plastic in, the frequently dense edge and the dense linings of the tubules apparently constitute the entire structure. When the plastic is removed, the external "membrane" is seen to be a dense peripheral portion of tubule wall and the tubules are seen to consist of both dense and less dense material. A sac-like membrane,[1] such as seen in Figures 4, 5, 9, surrounding the macronucleus, cannot be demonstrated, although the presence of the macronuclear membrane itself indicates that toluene treatment did not remove any structure like it.

The relation of this tubular interpretation of mitochondrial structure in *Paramecium* to the descriptions given for certain other organisms is not direct, although certain similarities are recognizable. Glimstedt and Lagerstedt (1953) conclude from studies of isolated particles that in rat liver the mitochondrion consists of a collection of granules arranged in cords; and although there is no evidence that the cords are hollow (tubular), the method of preparation would not permit observation of a cavity even if it does exist, and these structures may, in fact, be quite similar to those described for *Paramecium*. In the case of the mitochondrion of the germ cell of *Helix* (Beams and Tahmisian, 1954), which consists of a group of concentrically arranged lamellae, the relationship is even less clear and direct. However, the development of a lamellar structure from tubular precursors is suggested by Leyon (1954) for the development of the grana of the choroplast in *Aspidistra* from the primary grana. A process like this (phylogenetically or

[1] We are distinguishing between boundary interfaces and the kind of structure demonstrated by cell and nuclear membranes.

ontogenetically) could unite conceptually such seemingly diverse mitochondrial microanatomy as that seen in *Paramecium, Helix,* and the rat. On the other hand, it is recognized that real morphological differences in mitochondrial structure may exist; for instance, the mitochondrion of the proximal convoluted tubule as interpreted by Sjöstrand and Rhodin (1953) might suggest another basic structure.

The observations of Weinstein (1954) of the structure of particulates called sarcosomes in sections of chicken heart muscle are directly comparable to those reported here. The sections are quite similar to those of *Paramecium* mitochondria from which the plastic was not removed. Longitudinal and cross-sections of tubules within these particles in the muscle tissue are easily observed in the photographs. Although Weinstein (as well as Leyon) interprets his photographs as showing sac-like membranes, they can as easily be interpreted as not demonstrating the presence of a membrane on the external surface. This particle of the chicken heart, then, becomes nearly identical in structure to the mitochondrion of *Paramecium.*

Based on chemical and physical observations made on the mitochondria of several organisms, certain inferences concerning structure appear in the literature. One opinion is that the behavior of mitochondria in hypertonic and hypotonic solutions, together with some information on permeability of sarcosomes, indicate the presence of a differentially permeable membrane about the mitochondrion. This opinion was most recently presented by Lindberg and Ernster (1954) who feel that the recent morphological evidence as presented by Palade (1953) and Sjöstrand and Rhodin (1953) is complete confirmation of the membrane theory, and is so final as to make any contrary opinion "hardly maintainable today." Another view (Harman, 1950; Green, 1952) is that the biochemical and biophysical evidence is not of such a nature as to require that mitochondria possess membranes. It had been proposed that a complex protein can exhibit the osmotic behavior observed with no unique requirement for fluid interiors surrounded by differentiated membranes. The evidence presented in this paper supports this suggestion, in that the mitochondrion is shown to consist of an almost continuous material which is not separated from the exterior by a membrane different from it. One other characteristic, not predictable from the biochemical evidence, is revealed—the continuous material is the substance of the walls of tubules, the lumens of which are sometimes seen to open to the outside.

The way in which the mitochondrion functions physiologically may only be guessed from the morphological evidence. The most simple thought is that reaction of enzyme and substrate occurs at the external surface of the mitochondrion. However, the existence of the tubules and their openings to the cytoplasm suggests that these reactions may occur within the lumens of the tubules. In this instance, the surface available for reaction is very much greater than in the former case; and if in addition the peritubular spaces are involved, the surface reaction area is even greater yet.

Summary

1. An electron microscopical study of the mitochondria in *Paramecium aurelia* and *P. bursaria* has been made from thin sections of cells imbedded in methacrylate

plastic. Some of these sections were studied directly (plastic-in), and others were soaked in toluene before examination (plastic-out).

2. Plastic removal reveals structural detail not visible when the plastic remains in the section. At the same time, the relationship between the newly observed structures and those seen in the other sections is accounted for.

3. The undifferentiated mitochondrion in *Paramecium* is interpreted to consist of a compact mass of twisted tubules, the walls of which are made up of at least two kinds of substances (based on density differences in the photographs). The lumen of the mitochondrial tubule is about 15 mμ in diameter and is seen at times to be continuous with the cytoplasm. A distinct feature of this mitochondrion is the lack of a demonstrable membrane distinct from the material of the walls of the tubules in contrast to some interpretations of mitochondrial structure in many other organisms.

4. The basic tubular structure of the mitochondrion in *Paramecium* is recognizable in the cytoplasmic inclusions (mitochondria, primary grana) of several phylogenetically distant organisms.

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